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# TWO-ORGANISM CONCEPT FOR THE CONVERSION OF CELLULOSIC FEEDSTOCKS TO FUEL

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RESEARCH AND TECHNOLOGY DIRECTORATE

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The goal of this work was to investigate the potential of an anaerobic nitrogen-fixing, hydrogen-generating bacterial culture (cpnit-1 variant of *Clostridium phytofermentans*) coupled with a carbon dioxide- ($CO_2$) fixing, oil-generating algae culture (*Chlorella vulgaris*). In the anaerobic fermentation step, cpnit-1 produced 1.124 mol of hydrogen, 1.41 mol of $CO_2$, and 0.5 mmol of non-cellular organic ammonia (most of the ammonia was presumably incorporated into cells for growth). The production of ethanol, expected to be low under hydrogen-generating conditions, was measured as 3.53 µmol ethanol per mole of glucose consumed. The *C. vulgaris* algae culture subsequently assimilated, within detection limits, all of $CO_2$ produced by the bacteria and yielded 1.15 mol of oxygen per mole of glucose consumed. Various approaches were tested to purify the oil; the highest yield of algae oil was obtained using an isopropanol, water, and hexane mixture for extraction. To our knowledge, this represents the first demonstration of a hydrogen and oil-producing biological system capable of fixing its own nitrogen and $CO_2$. This approach offers advantages in terms of mass balance and environmental impact.

**15. SUBJECT TERMS**

| | | | | |
|---|---|---|---|---|
| *Clostridium phytofermentans* | *Chlorella vulgaris* | Alternative fuels | Ethanol | Carbon capture |
| Cpnit-1 | Biofuels | Hydrogen | Biodiesel | Carbon dioxide |

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# PREFACE

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## 1. INTRODUCTION

Since the start of the Industrial Revolution in the late 19th century, atmospheric levels of the greenhouse gas carbon dioxide ($CO_2$) have increased almost 31%, largely due to the combustion of fossil fuels and land-use changes such as forest clearing. In all, 480 PgC (1 PgC = $10^{15}$ g Carbon) have been released into the atmosphere since the start of the Industrial Revolution (Malhi et al., 2002). Figure 1 shows the obvious historical relationship between $CO_2$ levels and global temperature. This correlation is now a significant driver of industrial and public policies (Retallack, 2002). Photosynthetic organisms, because of their ability to assimilate atmospheric $CO_2$ and reduce it to chemical groups needed for energy and growth, have the potential to impact this trend through the production of carbon-neutral fuels from renewable materials (Wolosiuk et al., 1993).

The production of energy from renewable sources can serve to increase U.S. energy security and position the DoD to meet any future carbon accountability requirements. Toward this end, the U.S. Air Force already has a goal to acquire 50% of its fuels from a domestic source by 2016. Many DoD installations, especially in the Eastern United States, also have the potential to produce renewable, carbon-neutral energy from biomass.

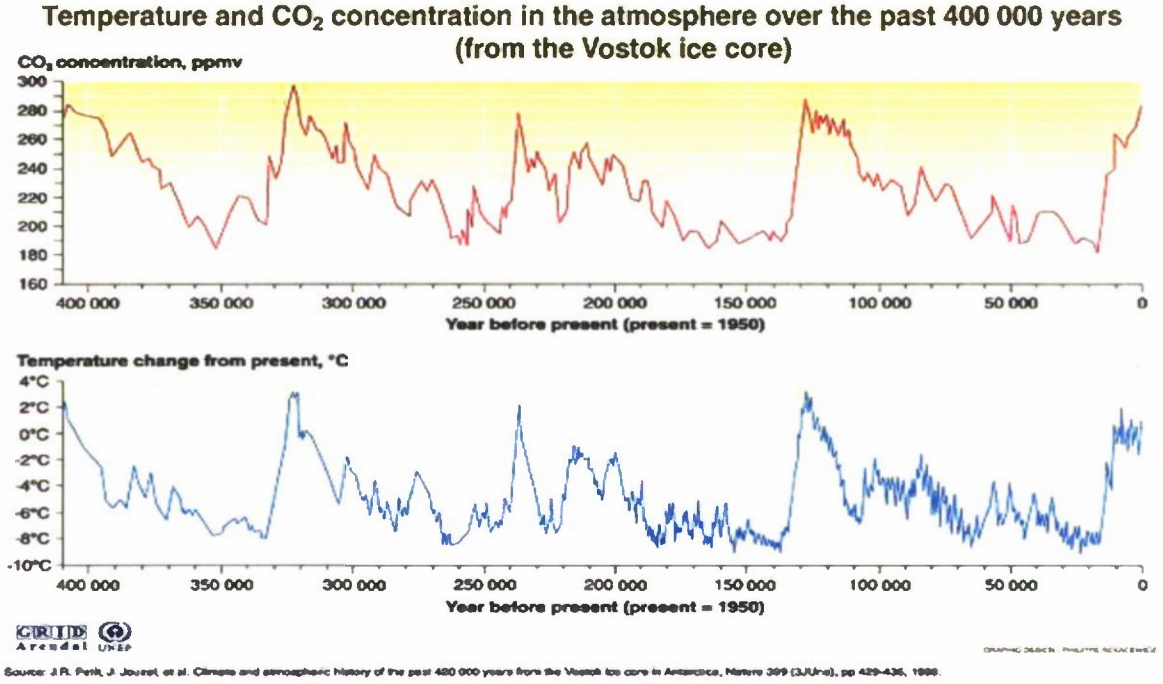


Figure 1. Relationship between Atmospheric $CO_2$ Levels and Temperature (Petit et al., 1999).

Hydrogen is an ideal fuel because its only oxidation product is water. When used in a fuel cell to generate electricity, it is three times as efficient as an internal combustion engine. However, its production, primarily from steam reformation of natural gas at 700-1100 °C ($CH_4 + H_2O \rightarrow CO + 3H_2$), requires much more energy than is created. Hydrogen can also be produced by electrolysis, splitting water into its component gases, hydrogen and oxygen. The electrical demand for that process far exceeds the energy value of the resulting hydrogen. Biological hydrogen production, typically using photosynthetic algae or anaerobic bacteria (dark fermentation), is an ambient temperature, catalytic process with the potential for a significant net energy gain. In order to be scalable, the process must be stable and to be economical and sustainable, it must provide a high yield of hydrogen from renewable feedstocks.

Biological nitrogen fixation provides about 40% of the nitrogen found in the world's soil and water (Postgate, 1998). Relatively few (perhaps 100) bacteria possess this capability in which atmospheric nitrogen ($N_2$) is reduced to ammonia via the nitrogenase enzyme. The reaction is of great economic importance as fully 1% of the world's energy supplies are consumed in the industrial fixation of nitrogen (Haber-Bosch process), mostly to produce fertilizer. Nitrogenase provides a catalytic alternative to the commercial fixation of nitrogen at a time when fertilizer has increased tremendously in price, primarily due to the cost of the natural gas from which it is made.

Nitrogenase also produces hydrogen in addition to ammonia (1 mol of $H_2$ per mole of $N_2$ fixed). A nitrogen-fixing, hydrogen-producing strain offers the potential to link these two reactions, thereby positively impacting the economics of both processes. Combined with a $CO_2$-fixing organism such as algae, the process could also capture the $CO_2$ it produces and convert it into oil to be processed into biodiesel. This kind of hydrogen- and oil-producing, nitrogen- and $CO_2$-fixing process, if employed on a large scale, could have a significant positive impact on the balance of carbon and nitrogen in the biosphere.

The objective of this work was to determine the extent to which both hydrogen and algae oil could be produced under carbon neutral, nitrogen-fixing conditions using a unique two-organism fermentation system and renewable feedstocks as starting material. The two-organism system consists of cpnit-1 (a variant of the anaerobic bacterium *Clostridium phytofermentans*) and the algae *Chlorella vulgaris*.

*C. phytofermentans* is a recently discovered anaerobic bacterium that produces ethanol, acetate, $CO_2$, and hydrogen (Warnick, 2002). Our lab isolated a variant strain of *C. phytofermentans*, cpnit-1, that has increased growth rate, produces significantly more hydrogen than the parental strain, and fixes its own nitrogen.

*C. vulgaris* is a freshwater, single-celled green microalgae that contains chlorophyll a and b pigments. As with all plants, *C. vulgaris* assimilates atmospheric $CO_2$ and converts it into compounds needed for plant growth and development. A significant portion of the carbon from $CO_2$ is converted into oil, which is suitable for processing into biodiesel. By sparging the $CO_2$ effluent from the bacterial fermentation

through a *C. vulgaris* culture, it becomes possible to recycle the $CO_2$ effluent from the bacterial culture into oil.

## 2. MATERIALS AND METHODS

### 2.1 Cpnit-1 and Green Algae Growth for Gas Measurement

#### 2.1.1 Bacterial and Algal Strains

Variant strain cpnit-1 was isolated in our lab from the parent strain, *C. phytofermentans* (American Type Culture Collection [Manassas, VA]). *C. vulgaris* was obtained from the University of Texas at Austin's Culture Collection of Algae (Austin, TX).

#### 2.1.2 Medium for Cpnit-1

A sample of cpnit-1 was grown under anaerobic conditions in 5 L New Brunswick Bioflo 100 5 L fermenters. Bacterial growth medium M1 consisted of (concentrations in grams per liter): glucose (1.0), $K_2HPO_4$ (2.9), $KH_2PO_4$ (1.5), adenine (0.02), cytosine (0.05), guanosine (0.02), uracil (0.04) and thymine (0.05), pH 7. The medium was supplemented with sterile vitamin and mineral solutions. The final concentrations of vitamins were the following (concentrations in micrograms per liter): biotin (20.0), folic acid (20.0), pyridoxine-HCl (100.0), thiamine-HCl (50.0), riboflavin (50.0), nicotinic acid (50.0), calcium panthothenate (50.0), $B_{12}$ (0.10), p-aminobenzoic acid (50.0), and thioctic acid (10.0). The final concentration of the mineral solution was the following (concentrations in milligrams per liter): sodium citrate (5.0), $MgSO_4 \cdot 7\ H_2O$ (62.0), $MnSO_4 \cdot 4\ H_2O$ (5.5), NaCl (10.0), $FeSO_4 \cdot 7\ H_2O$ (1.0), $CoCl_2 \cdot 6\ H_2O$ (1.7), $CaCl_2 \cdot 2\ H_2O$ (1.3), $ZnSO_4 \cdot 7\ H_2O$ (1.8), $CuSO_4 \cdot 2\ H_2O$ (0.5), $AlK(SO_4)_2 \cdot 12\ H_2O$ (0.5), $H_3BO_4$ (0.1) and $NaMoO_4 \cdot 2\ H_2O$ (0.11).

#### 2.1.3 Medium for *C. vulgaris*

*C. vulgaris* was grown under anaerobic, $CO_2$-fixing conditions in the following algal growth medium (concentrations in grams per liter): $KNO_3$ (5.0), $KH_2PO_4$ (1.25), $K_2HPO_4$ (0.1), $MgSO_4 \cdot 7\ H_2O$ (2.5) and NaCl (1.8), pH 6. This medium was sterilized and supplemented with 1 mL/L of $A_5$ solution and 1 mL/L iron solution. The $A_5$ solution consisted of the following (concentrations in grams per liter): $H_3BO_3$ (2.86), $MnCl_2 \cdot 4\ H_2O$ (0.22), $CuSO_4 \cdot 5\ H_2O$ (0.08) and $NaMoO_4 \cdot 2\ H_2O$ (0.021). The iron solution consisted of the following (concentrations in grams per liter): $FeSO_4 \cdot 7\ H_2O$ (25.0) and EDTA (33.5).

2.1.4 Hydrogen and $CO_2$ Gas Output from Cpnit-1 Monitoring

Five liters of sterile M1 medium was flushed for several hours with nitrogen gas to establish anaerobic conditions. Fermenters were inoculated with a small amount of cpnit-1 culture and temperature was maintained at 34 °C and the pH was kept constant at 7.00 with eoneentrated NaOH. Nitrogen gas flow was maintained at 20 mL/min. The hydrogen contcnt of the effluent gas was measured using hydrogen sensors ($H_2$Sean [Valencia, CA]). The $CO_2$ content of the gas effluent was measured using $CO_2$ monitors (Vernier Software and Teehnology [Eugene, OR]).

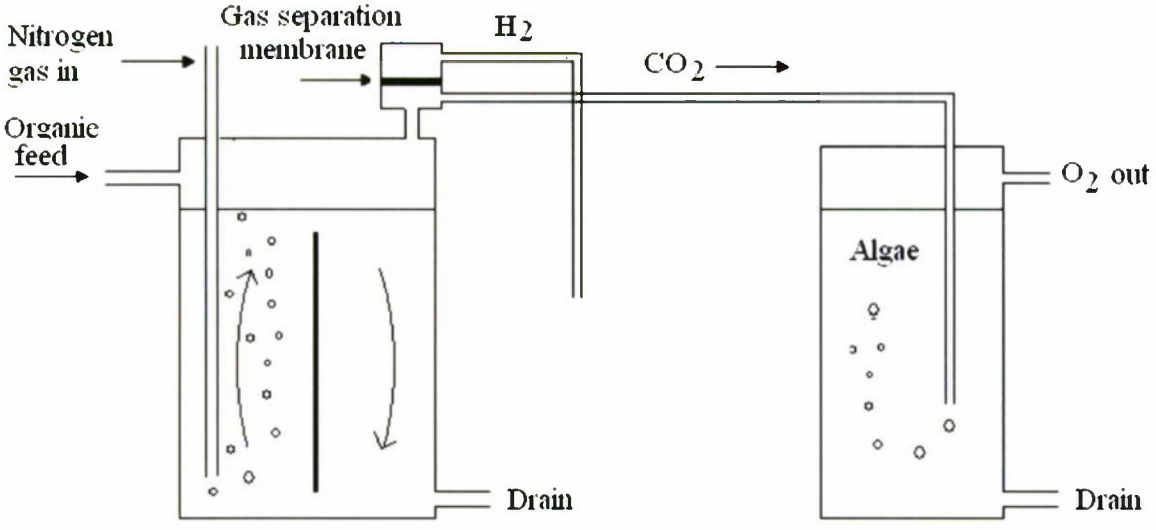


Figure 2. Coneept Diagram Illustrating Overall Proeess of Hydrogen Produetion in Baeterial Culture and Carbon Capture and Oil Produetion in Algae Culture.

2.1.5 $CO_2$ and Oxygen Gas Output from *C. vulgaris* Monitoring

Five liters of sterile M1 medium was flushed for several hours with nitrogen gas to establish anaerobic conditions. Fermenters were inoeulated with a small amount of epnit-1 eulture and temperature was maintained at 34 °C. The pH was kept constant at 7.20 with concentrated NaOH. Nitrogen gas flow was maintained at 30 mL/min.

Tubing was connected to the gas output of the fermenters containing epnit-1 and gas was passed through a 0.25 μm filter and then into a gas diffuser. The gas diffuser was plaeed into a flask containing 5 L of sterile algal growth medium (Figure 2). A 1 L eulture of *C. vulgaris* was added to the flask of algal growth medium. The flask was then moved into a growth ehamber where light was at a constant 1000 lux and temperature was 25 °C. The $CO_2$ produced by the cpnit-1 served as the sole carbon source for the algae. $CO_2$ and oxygen eontent out of the algae was measured using $CO_2$ and oxygen monitors (Vernier Software and Teehnology).

### 2.1.6 Quantification of Organic Ammonia Produced by Cpnit-1

A 50 mL sample of epnit-1 was removed from the fermenter and centrifuged at 3000 rpm for 25 min at room temperature. The supernatant was saved for analysis. A 10 mL aliquout of the supernatant was analyzed using the Hach Nitrogen/Ammonia Salicylate Method kit (Hach [Loveland, CO]).

### 2.1.7 Quantification of Ethanol Produced by Cpnit-1

A 50 mL sample of epnit-1 was removed from the fermenter and centrifuged at 3000 rpm for 25 min at room temperature. The supernatant was saved for analysis. The ethanol content in the supernatant was determined using the Ethanol Assay Kit (BioVision [Mountain View, CA]).

## 2.2 Algae Growth for Oil Extraction Measurements

### 2.2.1 Growth of *C. vulgaris*

Six liters of sterile algal growth medium was inoculated with 200 mL of *C. vulgaris*. The culture was stirred at room temperature using atmospheric $CO_2$ as the sole carbon source. When culture had reached proper density, it was filtered through a Millipore filter and concentrated. The resulting filtrate was centrifuged at 10,000 rpm at 4 °C for 20 min. The resulting pellet was dried for 48–72 h. The dried pellet was weighed and stored at 4 °C until further use.

### 2.2.2 Extraction with Isopropanol, Hexane, and Water

The method of Markham et al. (2006) was followed with the following modifications. The lower phase of a solution of 55:20:25 isopropanol:hexane:water was used for extractions. Two hundred fifty milligrams of dried algae was homogenized with 2.5 mL of the lower phase of the solution described above and heated at 60 °C for 15 min. The sample was centrifuged at 3000 rpm at room temperature for 15 min. The resulting supernatant was saved and the pellet was washed with an additional 2.5 mL of solvent and heated at 60 °C for 15 min. The sample was centrifuged for a second time at 3000 rpm at room temperature for 15 min. The resulting supernatant was combined with the first supernatant and the pellet was again washed with an additional 2.5 mL of solvent and heated at 60 °C for 15 min. The sample was centrifuged at 3000 rpm at room temperature for 15 min. The resulting supernatant was combined with the first and second supernatants and the pellet was discarded. The supernatants were dried and the sample was reconstituted in 200 µL of Fatty Acid Assay Buffer. Extracted oil was then quantitated using BioVision's Free Fatty Acid Quantification Kit. Excess oil extractions were stored at 4 °C.

### 2.2.3 Extraction with Ethanol, Water, and Hexane

The method of Fajardo et al. (2007) was followed with the following modifications. Two hundred fifty milligrams of dried algae was homogenized with 2.5 mL of 96% ethanol. The sample was centrifuged at 3000 rpm for 15 min at room temperature. The resulting supernatant was saved and the pellet was washed with an additional 2.5 mL of 96% ethanol. The sample was centrifuged at 3000 rpm for 15 min at room temperature. The supernatant was combined with the supernatant from the first centrifugation step. The pellet was washed with an additional 2.5 mL of 96% ethanol. The sample was centrifuged at 3000 rpm for 15 min at room temperature. The supernatant was combined with the supernatant from the first two centrifugation steps and the pellet discarded.

A 50:50 solution of water:hexane was prepared. The volume of this solution was equal to the volume of the three combined supernatants. The combined supernatants were added to the water:hexane solution and mixed. Phases were separated and the organic phase (upper phase) was removed and dried. The dried pellet was reconstituted in 200 µL of Fatty Acid Assay Buffer. Extracted oil was then quantitated using BioVision's Free Fatty Acid Quantification Kit. Excess oil extractions were stored at 4 °C.

### 2.2.4 Extraction with Hot Isopropanol

Isopropanol was heated to 60 °C. Two hundred fifty milligrams of dried algae was homogenized with 2.5 mL of heated isopropanol. The sample was centrifuged at 3000 rpm for 15 min at room temperature. The resulting supernatant was saved and the pellet was washed with an additional 2.5 mL of heated isopropanol. The sample was centrifuged at 3000 rpm for 15 min at room temperature. The supernatant was combined with the supernatant from the first centrifugation step. The pellet was washed with an additional 2.5 mL of heated isopropanol. The sample was centrifuged at 3000 rpm for 15 min at room temperature. The supernatant was combined with the supernatant from the first two centrifugation steps and the pellet discarded. The combined supernatants were dried. The dried pellet was reconstituted in 200 µL of Fatty Acid Assay Buffer. Extracted oil was then quantitated using BioVision's Free Fatty Acid Quantification Kit. Excess oil extractions were stored at 4 °C.

## 3. RESULTS

### 3.1 Production of Hydrogen and $CO_2$ by Cpnit-1

Grown under nitrogen-fixing conditions, cpnit-1 produces 1.124 mol of hydrogen gas per mole of glucose consumed. It also produces $CO_2$ as a metabolic by-product of nitrogen fixation. Under the conditions previously described in this report, cpnit-1 produces 1.41 mol of $CO_2$ gas per mole of glucose consumed (Figure 3).

## 3.2 Fixation of $CO_2$ by *C. vulgaris*

The gases produeed by the cpnit-1 were funneled into a 6 L flask eontaining 5 L of *C. vulgaris*. As shown in Figure 3, the *C. vulgaris* fixes all the $CO_2$ produced by the epnit-1. Oxygen levels in thc eulture dropped to a minimum value of approximately 17.7% aftcr the system ran for 40.6 min. This drop in culture oxygen eoneentration also corresponds to a point when no detectablc hydrogen and minimal amounts of $CO_2$ were being produced by the cpnit-1 (data not shown). However, after 40.6 min, the algae culture began to produce oxygcn. For the entire process, thc *C. vulgaris* culture produeed 1.15 mol of oxygen per mole of glucose. In addition to oxygcn produetion, the *C. vulgaris* fixed all the $CO_2$ produeed from thc cpnit-1 (Figure 4).

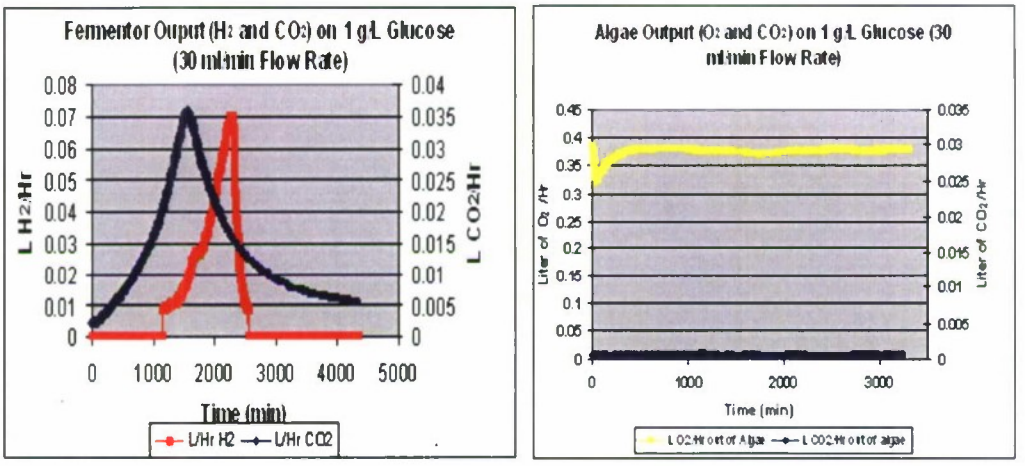


Figure 3. Hydrogcn and $CO_2$ Gascs Produeed by Cpnit-1 (left) and Oxygen and $CO_2$ Levels in the Headspace of the *C. vulgaris* Culture (right). To deteetable levels, all of the $CO_2$ produeed by thc bacterial culture was fixed by thc algae eulturc.

## 3.3 Ethanol and Organic Ammonia Produced by Cpnit-1

Cpnit-1 produccd 0.5 mmol of organic ammonia per mole of glucose consumed and 3.53 µmol of cthanol pcr mole of glucose consumed.

## 3.4 Algae Oil Extraetion

Thrcc diffcrent extraction methods were tested to determine and compare their efficiency of oil rccovery. The first method using an extraction of 55:20:25 isopropanol, hexane, and water resulted in a reeovery of 16.11 mg of algae oil from 250 mg of dried algae, corresponding to a 6.44% rccovery of oil from thc dry samplc. The second method using 96% ethanol followed by a 50:50 water and hexanc extraction resultcd in a recovery of 6.37 mg of algae oil from 250 mg of dried algac, corresponding to a 2.55% reeovery. The last method using hot isopropanol resulted in a

recovery of 4.5 mg of algae oil from 250 mg of dried algae, corresponding to a 1.8% recovery.

## 4. DISCUSSION

The carbon cycle is a complex process that results in the oxidation of energy-rich carbon containing compounds to $CO_2$ and the reduction of that $CO_2$ back into organic carbon (Ragsdale, 2007). Plants are an integral part of the carbon cycle, fixing atmospheric $CO_2$ and converting it into compounds that are essential for life and releasing oxygen as a by-product (Wolosiuk et al., 1993). Plants, with their ability to assimilate $CO_2$, can play an important role in reducing atmospheric $CO_2$ levels.

Rubisco (ribulose 1,5-bisphosphate carboxylase oxygenases, EC 4.1.1.39) is the key enzyme responsible for photosynthetic carbon assimilation, or $CO_2$ fixation. Rubisco is responsible for catalyzing the reaction between $CO_2$ and ribulose 1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglycerate (3PG). The 3-phosphoglycerate then enters the Calvin-Benson cycle and through a series of enzymatic steps is converted into glucose or it passes through the pentose phosphate pathway to produce other sugars. Eq 1 represents the net equation for the Calvin-Benson cycle.

$$6\ CO_2 + 18\ ATP + 12\ NADPH + 12\ H^+ + 12\ H_20 \rightarrow Glucose + 18\ P_i + 12\ NADP^+ \quad (1)$$

The energy produced in the Calvin-Benson cycle is then used to drive the light reactions of photosynthesis, which produces oxygen gas as a product. Eq 2 is the overall net equation of the Calvin-Benson cycle and the light reactions of photosynthesis:

$$6\ CO_2 + 6\ H_2O + photons \rightarrow Glucose + 6\ O_2 \quad (2)$$

Thus, the use of a plant-based carbon capture system (as described in this work), results in the next conversion of $CO_2$ gas to oxygen gas.

The work described in this report involves the use of cpnit-1, a variant strain of *C. phytofermentans*. Cpnit-1 produces significantly higher levels of hydrogen than the parent strain, *C. phytofermentans*. Although not described in this work, cpnit-1 can also produce hydrogen from various cellulosic feedstocks, using wastewater treatment plant effluent as the growth medium (Figure 4). In Figure 4, cpnit-1 was grown under nitrogen fixing conditions using Ammonia Fiber Explosion (AFEX) treated corn stover as the sole carbon source in wastewater treatment plant effluent and produced 1.0 mmol of hydrogen gas per gram of cellulosic feedstock.

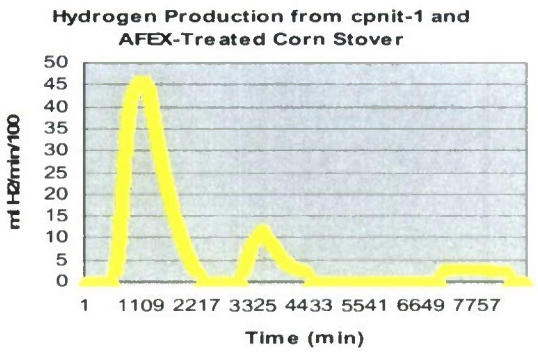


Figure 4. Hydrogen Production from Cpnit-1 and AFEX Treated Corn Stover as the Sole Carbon Source.

Plants (such as algae) and carbon-fixing bacteria assimilate approximately $10^{17}$ g of $CO_2$ per year; however, respiration releases a nearly equal amount of $CO_2$ back into the atmosphere (Ragsdale, 2007). *C. vulgaris* is a photosynthetic, unicellular algae. In this work, *C. vulgaris* was used to fix the $CO_2$ produced by cpnit-1 and to produce oil suitable for processing into biodiesel, another fuel of interest to the DoD, which can also be obtained from domestic, renewable sources.

This work demonstrates the production of hydrogen and algae oil (which can be processed into biodiesel) under carbon-neutral conditions using this two-organism system (Table). In addition to the two types of fuels, organic ammonia, which can be used as a nitrogen source for algae growth or a fertilizer, is also made during the production of the fuels.

Table. Overall Input and Output of the Two-organism System.

| Feed | Intermediate Products | Products |
|---|---|---|
| Plant Material (*Phragmites*, Switchgrass, etc.) | $CO_2$ | Bacteria (organic fertilizer) |
| Nitrogen gas | | Oxygen |
| Electricity for motors, pumps, etc. | | Algae (up to 50% oil) |
| Wastewater treatment plant effluent | | Water |
| | | Hydrogen |

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